

# ENGINEERING YEAST FOR PRODUCTION OF BENZOPHENONES AND XANTHONES AS PRECURSORS OF POLYCYCLIC POLYPRENYLATED ACYLPHLOROGLUCINOLS

Benye Liu<sup>1,2</sup>, Lars Fabian Kalz<sup>1</sup>, Yizhen Jia<sup>1</sup>, Marco Grull<sup>1,2</sup>, Till Beuerle<sup>1,2</sup>, Ludger Beerhues<sup>1,2</sup>

<sup>1</sup> Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Mendelssohnstrasse 1, 38106 Braunschweig, Germany; <sup>2</sup>Center of Pharmaceutical Engineering (PVZ), Technische Universität Braunschweig, Franz-Liszt-Strasse 35a, 38106 Braunschweig, Germany; email: [b.liu@tu-bs.de](mailto:b.liu@tu-bs.de)

## ABSTRACT

Polycyclic polyprenylated acylphloroglucinols (PPAPs) exhibit a broad range of biological activities, such as antidepressant, antibacterial, antiviral and antitumor properties. The content of PPAPs in the producing plants is often quite low, and their complex structures make total chemical synthesis difficult and economically impractical. Progresses in synthetic biology provide an alternative approach for the production of these valuable compounds. Here we present our results on reconstruction of the biosynthetic pathways of PPAP precursors in yeast. Based on our previous works on the biosynthesis of PPAPs, genes involved in the formation of benzophenones and xanthenes from *Hypericum sp.* and other organisms were expressed in yeast either episomally or by integration into the genome. The production of the expected products reached around 0.5 mg/l, which is high enough to be the substrate for enzymes of subsequent biosynthetic steps. The yeast strains will be further engineered by introducing various prenyltransferase enzymes to reconstruct the full biosynthetic pathways of PPAPs.

**Keywords:** benzophenone, xanthone, yeast, prenyltransferase, synthetic biology

## INTRODUCTION

Polycyclic polyprenylated acylphloroglucinols (PPAPs) are a group of structurally fascinating and synthetically challenging plant secondary products, which exhibit a broad range of biological activities, such as antidepressant, antibacterial, antiviral and antitumor properties [Yang, 2018]. The biosynthesis of PPAPs involves both the type III polyketide pathway and the terpenoid pathway. The acylphloroglucinol cores of PPAPs are produced via a characteristic type III polyketide biosynthesis process involving the condensation of one acyl-CoA and three malonyl-CoAs to form a tetraketide intermediate, which in turn cyclizes and aromatizes to yield acylphloroglucinols. Prenylation of this core structure and further cyclization of the prenyl side chains afford different PPAPs with diverse carbon skeletons. The type of acyl group, the number and position of isoprenyl residues, the degree of oxidation of isoprenyl side chains and the corresponding locations of ether rings as well as different types of secondary cyclizations (such as aldol, Diels–Alder, etc.) create PPAPs' structural diversity and complexity.

PPAPs have been exclusively isolated from plants of the families Hypericaceae and Clusiaceae, mainly from

the genera *Hypericum* and *Garcinia* with only few exceptions [Fiesel, 2015]. The content of PPAPs in the producing plants is often quite low, and their complex structure makes total chemical synthesis very difficult and in general economically impractical because of the low yield. Recent progresses in synthetic biology provide an alternative approach for the production of these valuable compounds. For example, artemisinic acid, the precursor of the antimalarial drug artemisinin, was successfully produced in engineered yeast cells with a yield of 25 g/l. Here we present our results of reconstruction of the biosynthetic pathways of benzophenone and xanthone in yeast.

## RESEARCH CONCEPT

**Plasmid construction and yeast strains:** *HpPKS006* is a type III polyketide synthase gene cloned from *Hypericum perforatum*, which has both benzophenone synthase (BPS) and isobutyrophenone synthase (IBS) activities [unpublished]. *RpBZL* is a benzoyl-CoA ligase gene from *Rhodopseudomonas palustris* [Egland, 1995]. *HcTXS* and *HcX6H* are xanthone synthase and xanthone 6-hydroxylase from *H. calycinum*, respectively [El-Awaad, 2016]. *HpPT8* is a xanthone prenyltransferase [Fiesel, 2015]. Yeast strains used are INVSc1 (Invitrogen), CEN.PK102-5B

[Entian, 2007] and DD104, which contains down-regulation of endogenous farnesyl pyrophosphate synthase, ERG20, by site-directed mutation of K197G, proven to provide more free IPP/DMAPP and GPP [Fischer, 2011, Li, 2015]. Genome integration was carried out using integrative EasyClone vectors with auxotrophic selection markers [Jensen, 2014].

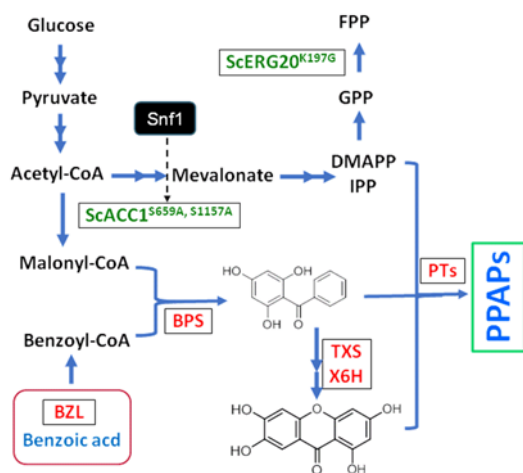


Fig. 1 Schematic illustration of the reconstruction of PPAP biosynthetic pathways in yeast. The endogenous yeast genes are in green, exogenous genes are in red.

**Reconstruction of benzophenone and xanthone biosynthetic pathways in yeast:** *HpPKS006* and *RpBZL* were subcloned into the vector pESC-URA (Agilent Technologies), *HcTXS* and *HcCPR* (cytochrome P450 reductase gene from *H. calycinum*) were subcloned into the vector pESC-LEU. *HcX6H* and *HpPT8* were subcloned into the vector pESC-HIS. The promoters of all the pESC vectors were changed to the constitutive promoter TEF1-PGK1.

Different combinations of the constructs were co-transformed into appropriate yeast strains using a high efficiency lithium acetate transformation protocol [Gietz, 2007]. The resulting positive yeast clones, after being verified by PCR, were cultured in 10 ml of synthetic dextrose dropout medium (-Leu, -His, -uracil) with D-glucose as the carbon source at 30°C and 200 rpm shaking for 36 hours. Then, the yeast cells were diluted into 20 ml of different media to OD<sub>600</sub> 0.05 and cultured at the same conditions. Benzoic acid was added at different time points with a final concentration of 3.5 mM. One ml of yeast cultures were taken at different time points, extracted twice with the same volume of ethyl acetate. Extracts were combined and dried under a stream of nitrogen, the residues were dissolved in 50% (v/v) methanol for HPLC-DAD

analysis. The product peaks were collected from HPLC and analyzed by LC-MS. The content of target compounds in the samples was quantified based on calibration curves of authentic references.

## RESULTS

**Selection of culture medium:** Among the three common yeast culture media, YPD is the best for both growth and product formation (Table 1). There are no significant differences between episomal expression and genome integration of the pathway genes.

Table 1 Kinetics of growth and benzophenone production in YPD, YPGE and SGI media

Medium	Strain	$\mu_{\max}$ (h <sup>-1</sup> )	OD <sub>30h</sub> (rel. AU)	r ( $\mu\text{g}^{-1}\text{h}^{-1}$ )	C <sub>max</sub> ( $\mu\text{g l}^{-1}$ )
YPD	genome	0.389	16.60	12.5	180
	episomal	0.386	13.62	12.8	230
YPGE	genome	0.195	3.24	1.7	60
	episomal	0.190	3.50	1.1	40
SGI	genome	0.290	3.14	0	0
	episomal	0.250	2.80	0	0

**Production of expected compounds:** The engineered yeast strains produced the expected products at a level of 0.1 mg/l without feeding benzoic acid in yeast strain INV.Sc1. Feeding exogenous benzoic acid increased the 2,4,6-trihydroxybenzophenone yield by a factor of 4 (Fig. 2). In contrast, yeast strain DD104 produced product only without feeding benzoic acid. Adding benzoic acid to a final level of 0.15 mM abolished product formation completely (data not shown).

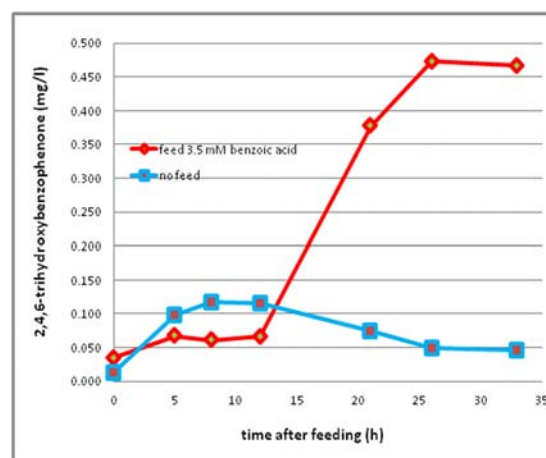


Fig. 2 Feeding benzoic acid increased the production of 2,4,6-trihydroxybenzophenone in yeast strain INV.Sc1 episomally over-expressing *HpPKS006* and *RpBZL* and grown in flask cultures.

## DISCUSSION

Here we successfully reconstructed the benzophenone and xanthone biosynthetic pathway in yeast using constitutive promoters, which simplify the production procedure by eliminating the induction step. There was no significant difference between the episomal expression and the genome integration in terms of both growth and product formation, indicating that the pathways are independent of the copy number of the genes. A similar result has also been found in the production of resveratrol in engineered yeast [Li, 2016]. The formation of benzophenone and xanthone proceeded by a growth-dependent manner, which has also been reported for resveratrol engineering works [Vos, 2015].

Although there is no information about benzoic acid metabolism in yeast, the fact that the engineered yeasts produce benzophenone and xanthone without feeding benzoic acid indicates that endogenous benzoic acid exists in yeast. The engineered benzophenone and xanthone biosynthesis process in yeast is a *de novo* production system. Further modification of the benzoic pathway may be helpful for increasing the product yield.

Co-expressing *HpPT8* in the xanthone producing yeast strain failed to produce detectable amounts of prenylated xanthone, indicating that more optimization works are needed to enable the correct function of prenyltransferase activity in yeast, as shown previously by bitter acid biosynthesis in yeast [Li, 2015].

## CONCLUSIONS

Engineered yeast strains produce the core structures of PPAPs at yields of around 0.5 mg/l. This amount is sufficient to serve as substrate for the downstream prenyltransferase enzymes, which have *K<sub>m</sub>* values at approx. 200  $\mu$ M. Thus, the strains so far generated can serve as the host strains for further reconstructing the complete pathways of PPAPs, which then may be available in sufficient quantities for the detailed assaying of their biological activities.

## ACKNOWLEDGMENT

The authors thank Dr. Irina Borodina from the Technical University of Denmark for providing the EasyClone vectors and helpful discussions. Thanks are

also given to the Bachelor students David Quast and Henning Ummethum for their efforts to over-express PKS006 in yeast.

## REFERENCES

- Egland, P.G., Gibson, J. and Harwood, C.S., 1995, Benzoate coenzyme A ligase, encoded by BADA, is one of 3 ligases able to catalyze benzoyl-coenzyme A formation during anaerobic growth of *Rhodopseudomonas palustris* on benzoate. *J. Bacteriol.*, Vol. 177, pp. 6545–6551.
- El-Awaad, I., Bocola, M., Beuerle, T., Liu, B. and Beerhues, L., 2016, Bifunctional CYP81AA proteins catalyse identical hydroxylations but alternative regioselective phenol couplings in plant xanthone biosynthesis. *Nat. Commun.*, Vol. 7, pp. 11472.
- Entian, K.D. and Kötter, P., 2007, Yeast genetic strain and plasmid collections. *Method Microbiol.*, Vol. 36, pp. 629–666.
- Fiesel, T., Gaid, M., Müller, A., Bartels, J., El-Awaad, I., Beuerle, T., Ernst, L., Behrends, S. and Beerhues, L., 2015, Molecular Cloning and Characterization of a Xanthone Prenyltransferase from *Hypericum calycinum* Cell Cultures. *Molecules*, Vol. 20, pp. 15616–15630.
- Fischer, M.J., Meyer, S., Claudel, P., Bergdoll, M. and Karst, F., 2011, Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol Bioeng.*, Vol. 108, pp. 1883–1892.
- Gietz, R.D. and Schiestl, R.H., 2007, High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.*, Vol. 2, pp. 31–34.
- Jensen, N.B., Strucko, T., Kildegaard, K.R., David, F., Maury, J., Mortensen, U.H., Forster, J., Nielsen, J. and Borodina, I., 2014, EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. *FEMS Yeast Res.*, Vol. 14, pp. 238–248.
- Li, H., Ban, Z., Qin, H., Ma, L., King, A.J. and Wang, G., 2015, A heteromeric membrane-bound prenyltransferase complex from hop catalyzes three sequential aromatic prenylations in the bitter acid pathway. *Plant Physiol.*, Vol. 167, pp. 650–659.
- Li, M., Schneider, K., Kristensen, M., Borodina, I. and Nielsen, J., 2016, Engineering yeast for high-level production of stilbenoid antioxidants. *Sci Rep.*, Vol. 11, pp. 36827.
- Vos, T., de la Torre Cortes, P., van Gulik, W.M., Pronk, J.T. and Daran-Lapujade, P., 2015, Growth-rate dependency of *de novo* resveratrol production in chemostat cultures of an engineered *Saccharomyces cerevisiae* strain. *Microb Cell Fact.*, Vol. 14, pp. 133.

Yang, X.W., Grossman, R.B. and Xu, G., 2018, Research Progress of Polycyclic Polyprenylated Acylphloroglucinols. *Chem Rev.*, Vol. 118, pp. 3508-3558.